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(19) Japan Patent Office
(12) Public Patent Disclosure Bulletin
(11) Public Patent Disclosure Bulletin No.: S57-109797
(43) Public Patent Disclosure Date: July 8, 1982

(51) Int. Cl.[3]	Identification code	Internal file numbers
C 07 G 7/06		6959-4H

Number of Inventions: 1
Request for examination: Not requested yet
(5 pages total)

(54) A method for preparing a keratin substance with a low molecular weight

(21) Application No.: S55-187190
(22) Application Date: December 26, 1980

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Specification

1. Title of the Invention

A method for preparing a keratin substance with a low molecular weight

2. Scope of Patent Claims

1. A method for preparing a keratin substance with a low molecular weight, wherein when the keratin substance is reduced and then hydrolyzed, a reducing reaction is applied to said keratins while the state of the raw materials in the reaction medium is maintained before and after the reducing reaction, and then, a hydrolysis reaction is applied in the water using a protein hydrolase.

3. Detailed Description of the Invention

The present invention relates to a method for preparing soluble keratins with a low molecular weight (this is called "oligokeratin" in the present specification); wherein structural protein that comprises wool, down, hair, etc., is used as a raw material and the range of the molecular weight is from 200 to 10,000. To date, it is well known that low molecular weight protein (oligopeptides) obtained by partial hydrolysis of fibrous protein such as collagen, keratins, etc., is useful as a cosmetic base for the hair, etc. (see I. Bonadeo, et al., Cosmetics and Toiletries, 92, 45 (1977)). Partial hydrolysis by acid or alkali, degradation by protein hydrolase, and a method wherein reflux extraction is done at high temperature (thermal decomposition) with a liquid medium have been employed as methods for preparing these oligopeptides. Since it is particularly easy to control the molecular weight of the oligopeptides obtained with the method that employs hydrolase, its usefulness is widely known in the field of protein chemistry.

However, in the event that a keratin substance is used as a raw material, the enzymatic degradation the is rooted in the chemical structure thereof and the high level tissue structure is difficult, so the enzyme processing method that is well known in keratin chemistry results in a high cost for the product since an extremely complex operation is required for the separation and recovery of oligokeratins, and it is not something that is satisfactory in practical use. That is, keratins are insoluble since they are equipped with a natural cross-linked structure (disulfide bonds, ion cross-linking, hydrogen bonds, hydrophobic interactions, etc.), and moreover it is characteristically difficult to hydrolyze them with enzymes. In the case of wool, the proportion in which untreated wool is degraded by pronase is 20% or less, the proportion degraded by tripsin is 10% at the highest, and the proportion degraded by pepsin is about 5% (see P.H. Springel, Aust. J. Biol. Sciy., 16, 272 (1965), W.G. Crowther, et al., Advan. Protein Chem., 20, 191 (1965), W.G. Crowther, et al., J. Biol. Chem., 242 (1967)). Therefore, to date a method is known wherein the disulfide bond that makes the keratins insoluble is cleaved by the action of a reducing agent in the presence of a dissolution aid such as urea, and causing the enzyme to act once the keratins have been dissolved in the liquid medium, as a method for adjusting oligokeratins with hydrolase. However, according to this kind of method, a great deal of effort is required for elimination dissolution aids such as urea and the reducing agents by separation by filtration, ultrafiltration, dialysis, etc., of the undissolved part at the time of reduction by filtration.

As a result of earnest examination aimed at solving these problems the present inventors arrived at the present invention by discovering the fact that it is possible to obtain oligokeratins that are readily soluble in water and organic polar solvents in a manner that is extremely easy operationally and in high yields by just causing protein hydrolase to act on fibrous reduced keratins (keratine) that have been treated by reduction, without destroying the form of the raw materials, for example the shape of the raw wool.

That is, the present invention provides a method for preparing a keratin substance with a low molecular weight, wherein when the keratin substance is reduced and then hydrolyzed, a reducing reaction is applied to said keratins while the state of the raw materials in the reaction medium is maintained before and after the reducing reaction, and then, a hydrolysis reaction is applied in the water using a protein hydrolase.

The purpose of the present invention is to provide a new method for preparing oligokeratin that possesses superior performance as a cosmetic base, but the advantages and features of the present invention will probably become clear from the following explanation.

The characteristics of the present invention are that (1) a natural substance comprising keratins as the main ingredient are reduced and the disulfide bonds in said keratins are cleaved, but the reducing reaction is performed under conditions where the keratin substance is present in a state where the form of the raw materials in the reaction medium is maintained after the reaction as well, and (2) oligokeratins are prepared with a high yield by causing hydrolase alone to act directly, without employing a swelling agent or dissolution aid for keratin substances that have been used to date during enzyme hydrolysis, on the reduced keratin substance wherein the form of the above-mentioned raw materials is maintained.

The advantages of the present invention are that (1) the separation of reduced keratins from the reducing reaction medium is extremely easy, since the reduced keratin substance is present in a state wherein the form of the raw materials is maintained, even in the reducing reaction medium, and (2) the enzyme hydrolysis reaction can be conducted in an aqueous solution that contains only an extremely small amount of acid or alkali, which has been added for the purpose of making the pH of the hydrolase employed optimal, and as a result it becomes possible to omit such complex processes as ultrafiltration and dialysis in the separation and recovery of the oligokeratins that dissolve in the aqueous solution, and the separation and recovery of oligokeratins becomes extremely easy.

The oligokeratins provided by the present invention can be items that generally have a molecular weight in a range of 200 to 10,000, and a molecular weight in range of 500 to 5,000 under optimal conditions. In addition, the above-mentioned oligokeratins possess a thiol side change produced as a result of the fact that that at least one part of the disulfide bone that forms the cross-link of the polypeptide chain of the keratins is cleaved, and in addition to the functional groups such as the amino group, carboxyl group, and alcohol and phenol hydroxyl group that the polypeptide chain possesses originally it also possesses an amino group and a carboxyl group on the molecular chain terminal, and thus is extremely rich in functionality and has a superior performance as a cosmetic base.

A more concrete explanation of the method of the present invention is provided below.

Keratins are one kind of structural (tissue) protein that is present in large quantities in the tissue that develops in the skin of vertebrates and towards the exterior thereof; therefore, one can cite the horns, hooves, claws, body hair, down, etc., of vertebrates, and preferably wool and down, as the keratin substances used as the raw materials in the method of the present invention, and it is possible to use these as is or by pulverizing or cutting them to an appropriate size in accordance with need.

In addition, it is also possible to carry out pretreatment, such as washing, degreasing, etc., of these keratin substances as need dictates.

According to the present invention, the above-mentioned keratin substances are first reduced. The reduction can generally be performed in a liquid medium. The liquid mediums that can be used must be stable towards reduction, and the selection of the reduction system medium is extremely important so that the keratin substance retains the form of the raw material thereof even after the reducing reaction. That is, it is necessary to select for the liquid medium that is used in the present invention something that does not dissolve or suspend keratins before and after the reducing reaction. Water that does not contain a dissolution aid, alcohols such as methanol, ethanol, n-propanol and isopropanol, or a mixture of water and an alcohol, are optimal as such mediums.

During reduction, the keratin substance of the above-mentioned raw materials is immersed in the above-mentioned liquid medium. At that time, there is no stringent restriction on the weight ratio of the keratin substance to the liquid medium, but it is best that to make this ratio at least 1: 20, and ordinarily a range of 1: 30 to 1: 100, in accordance with the kind, shape, etc., of the keratin substance that is used.

Any reducing agent that one wishes can be used as the reducing agent provided that it can cause the disulfide bond in the keratin substance (– 3 – 8 –) **[?; partly illegible]** to cleave to the thiol group (– 8H) **[?; partly illegible]**.

An organic and inorganic reducing agent of a type that acts neutrophilically towards the disulfide bond (– 3 – 8 –) **[?; partly illegible]** in general is preferable as a reducing agent that can be used. As the organic reducing agents, thiol derivatives and phosphorous compounds are suitable, and concretely such items as for example mercapto ethanol, mercaptoacetic acid, tributyl phosphine, triphenyl phosphine, etc., are used advantageously. In addition, such items as for example sodium hydrogensulfide ($NaHSO_3$) and sodium hydrosulfide (-NaSH) are optimal as the inorganic reducing agents.

The reduction itself can be carried out by the well-known methods (see W.G. Crowther, et al., Advan. Protein Chem., 20, 191 (1965), J.A. Maclaren, Aust. J. Chem., 15, 824 (1962), J.A. Maclaren, et al, Aust. J. Chem., 19, 2355 (1966)). For example, in the event that in the event that the above-mentioned thiol derivatives or inorganic reducing agents are used, it is advantageous that these are generally used in an excess equivalent, ordinarily in an equivalent of at least twice and preferably an equivalent of 4 to 10 times per one disulfide bond, relative to the disulfide bonds in the keratin substance used, and in addition in the event that the above-mentioned phosphorous compounds are used it is sufficient in general if these are used in an equivalent to a slightly excess equivalent relative to the disulfide bonds in the keratin substance used.

The amount of disulfide bonds in the keratin substance can be determined by amino acid analysis, and the method thereof is well known (M. Friedman, A.T. Noma, Textile Res. J., 40, 1073 (1970)).

The reduction can be performed under pH conditions of acidity, neutrality or alkalinity. However, swelling or dissolution of the keratin substances occur under somewhat high or low pH conditions, and it becomes impossible to retain the form of the raw materials of said reduced keratin substances, so ordinarily it is advantageous ordinarily to carry out the reduction in a pH range of 1 to 8.

There are no particular restrictions on the temperature and pressure during reduction, and it is possible to vary these over a wide range in accordance with the type of reducing agent and the type of raw materials used, but room temperature is adequate as the reducing temperature. The pressure during reduction and normal pressure are adequate, but it is also possible to carry out the reaction under reduced pressure or applied pressure in accordance with need.

In addition, the reduction can preferably be carried out in an inert gas atmosphere, for example in nitrogen.

By reduction like that described above, the disulfide bonds in the keratin substances are cleaved, but said keratin substances are present in a state wherein the form of the raw materials is maintained in the reducing medium. Therefore, it is possible to separate and recover the reduced keratin substances easily by such means as filtering and centrifugation, and to add the following enzyme treatment processes after the substances have been fully washed. During said separation operation and washing operation, it is necessary to pay attention so that the thiol side chains that are produced by the cleaving in the keratin substances are not oxidized in the separation operation, for example it is performed in an inert gas atmosphere, or it is preferable that the separation and cleaning operations are carried out in a steady state relative to the oxidation of said thiol side chains by adjusting the pH of said reducing medium or washing liquid to 6 or below.

The adjustment of the pH can be easily carried out by adding a water-soluble acidic substance, for example, inorganic acids such as hydrochloric acid, sulfuric acid, nitric acid, phosphoric acid, etc., or organic acids such as acetic acid, propionic acid, p-toluene sulfonic acid, etc. In particular, for reasons of ease of separation of the acid from the oligokeratins produced, a volatile acid, in particular hydrochloric acid, is preferable.

Enzyme hydrolysis treatment is then applied for the reduced keratin substances wherein the form of the raw materials obtained as described above is maintained. In the present invention, said enzyme hydrolysis is performed in an aqueous solution that is adjusted so that the inactivity of the hydrolase used becomes optimal, but it is one of the features of the present invention that said aqueous solution contains only a small amount of acid or alkali that is added in order to adjust the pH of the aqueous solution, and the hydrolase that is used, and this is also an advantage. It is possible to use an acidic enzyme such as pepsin and a neutral enzyme such as papain, pronase, tripsin, etc., as the above-mentioned hydrolase. However, the thiol side chains with reduced keratin are unstable when the pH of the aqueous medium is 6 or above and easily oxidized, and disulfide bonds are formed easily (see Shigeru **[illegible]**, "Chemistry of Organic Sulfur Compounds (Part 1)", *Kagaku Dojin*, Chapter 2, 1968). Therefore, it is desirable that hydrolysis be carried out with an acidic enzyme, preferably something like pepsin. In addition, it is necessary to restrict the temperature of the enzyme hydrolysis so that the enzyme that is used exhibits optimal activity. For example, a temperature range of 35 to 40 °C is preferable. The regulation of the molecular weight of the oligokeratins used can be carried out by selecting as appropriate the type, amount, reaction time and temperature of the enzyme that is used. In the event that deactivization of the enzyme used is necessary, this can be carried out by the methods that are well known in the field of enzyme chemistry, such as thermal treatment or pH adjustment.

In the event that insoluble foreign matter that adheres to the keratin and insoluble solid content is present, it is possible to carry out the separation and recovery of

oligopeptides from the oligopeptide aqueous solution that is obtained as described above by removing these by such means as filtration and centrifugation, after which an ordinary method is carried out such as for example freeze drying after said keratin aqueous solution is concentrated.

The oligokeratins obtains in this manner are white to light yellow powders, and it is possible to employ these for uses as a cosmetic base, and in particular a cosmetic base for hair.

As described above, when preparing oligopeptides, to date a refining process such as ultrafiltration or dialysis was indispensable, and thus a large plant for preparing oligopeptides was required on an industrial scale since said refining process was necessary, and a great deal of time was required as well, but the present invention does not require said refining process, nor does it require a large plant or a long time, and thus it possible for the first time to prepare oligopeptides with an extremely simple process.

A more concrete explanation is provided below of the present invention by citing working examples.

Working Example 1

10 g wool fiber was immersed in 1,000 cc of a 20% (W/W) n-propyl alcohol aqueous solution, and an adequate amount of nitrogen gas was caused to flow into the solution. Then, 2.5 ml tri-n-butyl phosphine was added, and then it was shaken lightly for 1 day at room temperature. At this point in time, the wool retained its original fibrous state, and after it was filtered and washed with ethanol, it was subjected to drying under reduced pressure at 30 °C, and fibrous reduced wool was obtained. 10 g fibrous reduced wool that was obtained in this manner was immersed in 1,000 cc 0.0075 N hydrochloric acid (pH 1.3), 200 mg pepsin was added and then it was shaken for 1 day at 40 °C, and the hydrolysis reaction was carried out. As the hydrolysis reaction progresses, the wool fibers dissolved in the hydrochloric acid, and 3 to 4 hours later there was almost no undissolved portion left. After the oligokeratin hydrochloric acid solution obtained in this manner was filtered, it was concentrated to approximately 300 cc with an evaporator, and 9.8 g of a light yellow powder oligokeratin was obtained by freeze drying (solubility rate 98%). Said oligopeptide was carboxymethylized with iodoacetic acid, and when its molecular weight was calculated by gel filtration (using G-50 Sephadex) it was 1,100. The molecular weights were calculated in the same manner in the following working examples.

Reference Example 1

10 g of untreated wool fiber to which reduction was not applied was immersed in 1,000 cc 0.075 N hydrochloric acid, and it was shaken for 1 day at 40 °C and a hydrolysis reaction was carried out. At this point in time, a majority of the fibrous wool remained, and when this was filtered, washed with ethanol and dried it was 9.2 g. In other words, the solubility rate was 8%.

Working Example 2

The same operation as in Working Example 1 was carried out, except for the fact that 15 ml mercaptoacetic acid was used as the reducing agent instead of the tri-n-butyl phosphine, and 1,000 cc water was used as the reducing reaction dispersion agent instead of the 20% n-propyl alcohol aqueous solution, in Working Example 1. The result thereof was that a light yellow powder oligokeratin with a solubility rate of 95% was obtained. Its molecular weight was 1,000.

Working Example 3

The same operation as in Working Example 1 was carried out, except for the fact that 15 ml 2-mercapto ethanol was used as the reducing agent instead of the tri-n-butyl phosphine, and 1,000 cc water was used as the reducing reaction dispersion medium instead of the 20% n-propyl alcohol aqueous solution, in Working Example 1. The result thereof was that a light yellow powder oligokeratin with a solubility rate of 90% was obtained. Its molecular weight was 1,000.

Working Example 4

The same operation as in Working Example 1 was carried out, except for the fact that 20 g sodium hydrogensulfide was used as the reducing agent instead of the tri-n-butyl phosphine, and 1,000 cc water was used as the reducing reaction dispersion medium instead of the 20% n-propyl alcohol aqueous solution, in Working Example 1. The result thereof was that a light yellow powder oligokeratin with a solubility rate of 92% was obtained. Its molecular weight was 1,100.

Working Example 5

The same operation as in Working Example 1 was carried out, except for the fact that the pH of the hydrolysis reaction medium was set at 6.8 by employing 200 mg papain as the hydrolase instead of pepsin in Working Example 1. The result thereof was that a light yellow powder oligokeratin with a solubility rate of 50% was obtained. Its molecular weight was 1,500.

Working Example 6

The same operation as in Working Example 1 was carried out, except for the fact that the pH of the hydrolysis reaction medium was set at 7.2 by employing 200 mg pronase P as the hydrolase instead of pepsin in Working Example 1. The result thereof was that a light yellow powder oligokeratin with a solubility rate of 85% was obtained. Its molecular weight was 1,500.

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